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NuMA interacts with phosphoinositides and links the mitotic spindle with the plasma membrane

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*Editor: Hartmut Vodermaier***Transaction Report:**

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

25 February 2014

Thank you again for submitting your manuscript on NuMA interaction with phospholipids to The EMBO Journal. It has now been reviewed by three expert referees, who all consider your findings of interest and potential importance. Nevertheless, they all raise a number of issues that would need to be satisfactorily addressed before eventual publication. In my view, the key points in this respect would be to strengthen the functional significance of NuMA cortical targeting by membrane binding (see ref 1 pts 1 & 5, ref 2 pt 4, ref 3 pt 1), and to assess possible effects/causal roles of disrupted cortical actin functions upon phospholipid perturbations (ref 2 pts 2 & 3, ref 3 pt 3). In addition, there are also several technical points and control issues that would need to be adequately addressed. On the other hand, I do not think it would be essential to experimentally follow up further on the aspect of CYK4-NuMA competition discussed by all three referees.

Should you be able to satisfactorily respond to these key points and the other more specific points detailed by the reviewers, we shall be happy to consider the study further for publication. Please keep in mind that it is our policy to allow only a single round of major revision, but also that competing manuscripts published during the three-months revision period will have no negative impact on our final assessment of your revised study; please nevertheless contact me as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting the three-month deadline, please let me know in advance and we could discuss the possibility of an extension.

Thank you again for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision!

REFeree REPORTS:

Referee #1:

The manuscript by Kotak et al. uses cultured human cells to interrogate the mechanism of NuMA targeting to the cell periphery in anaphase cells. NuMA/dynein are critical for spindle orientation and positioning in dividing cells. Recent work has suggested that NuMA is targeted to the cell cortex in anaphase by binding to either LGN/Galpha or 4.1 proteins (Kiyomitsu and Cheeseman, Cell 2013). This manuscript challenges these conclusions and proposes that NuMA directly associates with the plasma membrane for its targeting to the cell poles in anaphase. Using drug treatments and elegant chemical genetic manipulations, the authors provide strong evidence for the involvement of PI4P and PI(4,5)P2 lipids in membrane binding of NuMA. The experiments in the manuscript are generally well controlled and described. The identification and characterization of a membrane interaction module for anaphase recruitment of NuMA and its spatial control is timely and should be of interest to researchers in the areas of cell division and microtubule research. In the opinion of this reviewer, there are several critical points that should be addressed (see below) in order to support the model proposed by the authors.

Major comments:

- 1- Title: experiments probing the biological relevance of NuMA's interaction with the membrane would be important to support the title.
- 2- The role of CYK4 in preventing equatorial localization of NuMA in anaphase: have the authors attempted expressing the membrane interaction domain of CYK4 to test competition with NuMA membrane interaction? Also, have the authors tested whether assembly of the contractile machinery in general at the equator contributes to equatorial exclusion of NuMA?
- 3- The membrane localization of NuMA-mem is not clear from the picture in Fig3A. Quantification of peripheral association would be crucial to support the claim. Also, is the actomyosin cortex dispensable for the localization of NuMA to the envelope? This point is especially important given the authors claim that 4.1 proteins could contribute to NuMA localization via actin at the cortex.
- 4- This work suggest that contrary to recently published observations the 4.1 binding of NuMA may not be involved in its localization in anaphase. If so, localization of NuMA delta 4.1 in LGN RNAi cells would be predicted to be normal. This experiment would strengthen the authors' claims.
- 5- A key point that appears to be missing from this work is the biological relevance of NuMA's interaction with the membrane: is spindle positioning in anaphase affected by deleting the membrane association domain in a full length NuMA complementation system if e.g. LGN is depleted at the same time?

Minor comments:

- Page 13: PIP3 "... appears to be strictly in the cytoplasm ..."; it is equally or more likely that under the given condition cells have too little PIP3 in the membrane for it to be efficiently labelled by AKT-PH domains. I would recommend rephrasing the sentence.

Referee #2:

In this article Kotak and colleagues clarify how important molecular players are recruited to the cell cortex to generate forces on the mitotic spindle. They find, as was reported before, that the LGN pathway is dispensable for NuMA enrichment at the cortex during anaphase. They then clarify the previously published role of 4.1R and G proteins, showing that they in fact act indirectly on NuMA recruitment at the cell cortex, as their depletion has a general effect on the actin cortex. They propose that phosphoinositides have a more direct role in recruiting NuMA. They demonstrate a direct interaction in vitro and identify a small peptide in NuMA which is enough for membrane targeting. They propose that the spatial localization of NuMA in anaphase is also ensured by exclusion from the furrow region due to the presence of CYK4.

Overall, the experiments are convincing and the results are important for the field of spindle positioning and thus in general for cell division. I think the article could almost be published as it is. I just have a few minor comments.

- 1) An important point made by the authors is that 4.1G/R in fact act on cortical actin, which is itself required for NuMA recruitment at the cell cortex. But the images showing the actin cortex in

4.1(R+G) RNAi cells do not show such a drastic perturbation of the actin cortex. COuld the author show that using low doses of LatA which would have similar partial effect on actin, NuMA is already affected as much as with the 4.1(G+R) RNAi?

2) The authors propose an alternative mecanism for the recruitment of NuMA, but they do not show that the perturbations they do on PiPs do not affect cortical actin. Similarly to the effect of 4.1 depletion, perturbing PiPs is very likely to affect cortical actin too (in particular the perturbation via Ionomycin and Ca²⁺ is almost certain to strongly affect cortical actin)

3) Alternatively, do the authors suggest that the effect of cortical actin on NuMA is through PiPs? How do they reconcile the need for an intact actin cortex for NuMA recruitment and the role of PiPs?

4) The authors did not describe what is the phenotype of having NuMA all over the corex in anaphase. Is there an effect on spindle positioning or elongation?

5) The sentence on tumorigenesis in the discussion is a bit useless and could be removed.

6) The proposed mecanism of competition between CYK4 and NuMA for binding to PiPs is not very convincing. As they performed in vitro experiments for NuMA binding, maybe they could try their idea in vitro? (this is nevertheless absolutely not required for this article, as it is not a very central point).

Referee #3:

Summary

Understanding mitotic mechanisms that control cortical Dynein enrichment is of vital importance since they dictate spindle rotation, orientation and elongation processes. Prior to anaphase, Dynein is recruited to the cell cortex by an evolutionarily conserved platform - the LGN-NuMA-Galpha ternary complex. In contrast, during anaphase, NuMA and Dynein are recruited to the cortex independently of LGN and Galpha, but the underlying mechanism is unclear. In here, the authors present multiple lines of evidence to demonstrate that Phosphoinositide levels influence cortical NuMA levels in anaphase. They also identify the domain in NuMA that is responsible for physical interaction with phosphoinositides. Thus they present a novel mechanism for a direct link between astral microtubules and the plasma membrane. In addition, the authors have made sufficient contribution to clarify inconsistency in previous studies. However, they need to address a few points before drawing the conclusions in the manner presented.

Section IA. Specific major concerns (Points for data clarification):

1. Does the localization of cortical Dynein mirror the localization of NuMA mutants in Figure 1? In the image of the mutant expressing cell (Figure 1L and 1Q), is the level of NuMA dramatically reduced at spindle poles and increased at cortex - if so, how about dynein localization? This is important to address to fully understand the underlying biological significance.

2. In Figure 2C, is the NuMA (mem+C-ter) mutant excluded from the equatorial cell-cortex or not? The image shown looks ambiguous. This is important to conclude the domain responsible for NuMA exclusion from equatorial cortex.

3. Is it possible that altering phosphoinositide levels within the cell or cell-cortex disrupts cortical actin function and in turn, indirectly abrogates NUMA recruitment?

If this is technically difficult to disentangle, and not the scope of this paper, the authors should at least discuss this possibility because they are using an argument of altered actin function to negate some of the interpretations made by the Kiyomitsu and Cheeseman 2013 Cell paper and this could be true for their own model as well.

4. How are spindle oscillations calculated and how are they distinguished from tumbling? In the time-lapse sequence presented in Figures 5 and S4 and associated movies, spindle tumbling, but not

oscillation, is observed. It will be useful to measure pole-to-pole oscillation as a kymograph to compare oscillations across conditions. Also, statistical significance of differences between the s.e.m error bars in Figures 5E-G should be indicated using p-values (preferably shown as SD bars as in other figures).

Section IB. Specific major concerns (Points for text clarification):

1a. Results presented in the main Figure 1A-F are already known. It is already established that the LGN/Galpha platform is dispensable for cortical dynein recruitment in anaphase (eg., Kiyomitsu and Cheeseman, 2013). The authors could briefly clarify as to why they are presented as novel findings/ how these results extend current findings.

1b. Along the same lines, why do the authors consider their findings 'unexpected' in the discussion statement below: "Unexpectedly, we found here that the ternary complex components LGN/G α 1-3 are dispensable for NuMA-dependent enrichment of cortical dynein in the polar regions during anaphase"

2. Some of the panels in Figure-1 are not described in results text (see page 7) and it's confusing to the see these panels referred to in the later part of the manuscript.

3. I have two concerns regarding this statement: "In addition to uncovering a switch in the mechanism directing NuMA/dynein to the polar regions of the cell between metaphase and anaphase, we discovered an accompanying switch in the mechanism preventing their accumulation in the cortical equatorial region."

3a. Are the authors referring to their previous paper when they mention "In addition to uncovering a switch in the mechanism directing NuMA/dynein to the polar regions of the cell between metaphase and anaphase"? If yes, this should be referenced and made clear.

3b. The statement "we discovered an accompanying switch in the mechanism preventing their accumulation in the cortical equatorial region" is not fully supported here for the following reasons: The authors simply have a correlation between loss of CYK4 and accumulation of NUMA at equatorial cortex; and as they report they have a 'tempting possibility' that the two proteins may compete for same lipid moieties. If competition is indeed the full explanation for the switch, one would expect that NUMA overexpression displaces CYK4 from the equatorial cortex, but this has not been shown. Therefore, the sentence should be reworded as a proposal, rather than as discovery of a switch mechanism.

4. Toyoshima et al., 2007 reported metaphase-positioning defects in response to impaired PI3K activity. In this paper, phosphoinositides are reported to be responsible for anaphase recruitment of NuMA to cortex. Is there an LGN-Galpha independent pool of NuMA at the metaphase cell cortex? If so, could this be observed in Figures 1A-F?

Section II: Minor concerns that should be addressed

(i) Scale bars are missing in several images (examples: FigureS1, S3)

(ii) N values missing (examples: Figures 3, S4)

(iii) Typos: Page 5: 'indentify' instead of 'identify',

(iv) inconsistent nomenclature for s.e.m, Galphai(1-3)

(v) Incomplete sentence in methods:

"CDK1 inhibition was performed by treating metaphase synchronized cells for 5 minutes with either RO-3306 (Vassilev et al, 2006) (9 M; Santa Cruz, sc-358700)"

Point-by-point response to the reviewers

Reviewer #1

This reviewer recognized that our paper “...*should be of interest to researchers in the areas of cell division and microtubule research*”, but requested that several outstanding issues be addressed, which we have done as explained below.

Major Comments:

1. Title: experiments probing the biological relevance of NuMA's interaction with the membrane would be important to support the title.

Response. We respectfully disagree with the reviewer's implicit suggestion to change the title. We are of the opinion that the title is worded in a prudent manner, avoiding causality claims, even if only the experiments from the initial submission were to be considered. Furthermore, additional evidence supporting the importance of the interaction of NuMA with the plasma membrane has been provided in the revised manuscript, further substantiating the contents of the title (see response to point 4 below).

2. The role of CYK4 in preventing equatorial localization of NuMA in anaphase: have the authors attempted expressing the membrane interaction domain of CYK4 to test competition with NuMA membrane interaction? Also, have the authors tested whether assembly of the contractile machinery in general at the equator contributes to equatorial exclusion of NuMA?

Response. We agree with the reviewer that it would be interesting to further investigate the competition mechanism between CYK4 and NuMA. However, conducting such follow-up experiments appears to fall outside the scope of the present manuscript. However, as requested also by this reviewer, we have tested the contribution of the contractile machinery on NuMA exclusion from the equatorial region by using the Rho-Kinase inhibitor Y27632. These experiments establish that inhibiting the contractile machinery does not influence the equatorial exclusion of NuMA in anaphase. This new experiment is reported in Supplementary Figure S2E and S2F and discussed on p. 9 of the revised manuscript. In addition, to strengthen the finding of CYK4-dependent equatorial

exclusion of NuMA, we have tested the role of the kinesin protein MKLP1, which is known to be needed for CYK4 localization at the equatorial membrane. As shown in the new Supplementary Figure S2C and S2D and discussed on p. 8-9, we found that MKLP1 depletion also results in the presence of NuMA in the equatorial region, as upon CYK4 depletion. This further strengthens the notion that CYK4/MKLP1 outcompetes NuMA for binding to the equatorial cortical region. See also responses to point 6 of reviewer 2 and to point 2 of reviewer 3.

3. The membrane localization of NuMA-mem is not clear from the picture in Fig3A. Quantification of peripheral association would be crucial to support the claim. Also, is the actomyosin cortex dispensable for the localization of NuMA to the envelope? This point is especially important given the authors claim that 4.1 proteins could contribute to NuMA localization via actin at the cortex.

Response. As requested by the reviewer, we have performed quantification at the polar cortex of GFP-NuMA_{mem}, as well as of GFP-NuMA, GFP-NuMA_{c-ter} and GFP-NuMA_{mem+C-ter}. This quantification clearly demonstrates a minor, yet significant, enrichment of NuMA_{mem} at the cell cortex (see revised Figure 3 and associated legend on p. 44). In addition, we now report the distribution of GFP-NuMA_{mem} throughout mitosis in a new Supplementary Figure S3A-D that is mentioned on p. 9-10 of the revised text. These new pieces of data, together with the distribution of cortical NuMA_{mem} during interphase (see Figure 5 and 6), demonstrate that NuMA_{mem} can localize to the membrane throughout the cell cycle, in line with the likewise presence of PIP/PIP₂. As mentioned already in the initial submission, we stress again in the revised manuscript that the cortical distribution of GFP-NuMA_{mem} is weaker than that of full-length NuMA or of GFP-NuMA_{mem+C-ter}, suggesting that other regions contribute to anaphase cortical targeting. As for the comment regarding the role of the actomyosin cortex being dispensable: we may not have been sufficiently clear about this point, but our initial submission already contained this piece of data, demonstrating that F-actin contributes to cortical NuMA localization both in metaphase and anaphase (see Supplementary Figure S4A-L and accompanying text on p. 11 of the revised manuscript).

4. This work suggest that contrary to recently published observations the 4.1 binding of NuMA may not be involved in its localization in anaphase. If so, localization of NuMA delta 4.1 in LGN RNAi cells would be predicted to be

normal. This experiment would strengthen the authors' claims.

Response. We thank the reviewer for bringing up this point. As suggested, we have investigated the localization of GFP-NuMA $\Delta_{4.1}$ in cells that are depleted of LGN by RNAi. As shown in the new Supplementary Figure S4N and S4O and discussed on p. 12, depletion of LGN in anaphase cells does not affect cortical localization of GFP-NuMA $\Delta_{4.1}$, in contrast to its impact on metaphase cells. These results further demonstrate that NuMA cortical localization is not regulated by 4.1(R+G) proteins (nor LGN) during anaphase.

5. A key point that appears to be missing from this work is the biological relevance of NuMA's interaction with the membrane: is spindle positioning in anaphase affected by deleting the membrane association domain in a full length NuMA complementation system if e.g. LGN is depleted at the same time?

Response. Although this is an interesting experiment in principle, we reported previously that only ~50% of cells exhibit complete loss of NuMA cortical signal even after double treatment with siRNAs, and that such complete loss is necessary to observe defects in spindle elongation (see Materials and Methods section of Kotak et al., 2013; PMID: 16085494). Nevertheless, we attempted to perform the experiment requested by the reviewer, but found that the concomitant depletion of LGN by siRNAs, on top of the necessary double treatment with NuMA siRNAs and the transfection with GFP-NuMA Δ_{mem} , did not result in sufficient depletion of endogenous NuMA for a meaningful analysis of spindle elongation. Therefore, we decided instead to monitor the presence of cortical dynein in such cells because we and others have shown that cortical dynein drives spindle elongation during anaphase and is a more sensitive readout of NuMA depletion than the spindle elongation phenotype *per se* (see Kotak et al., 2013; PMID: 16085494, Kiyomitsu and Cheeseman, 2013; PMID: 23870127). As shown in the new Figure 3F-I and discussed on p. 10, we found that whereas GFP-NuMA can rescue the loss of cortical dynein incurred upon depletion of NuMA and LGN, GFP-NuMA Δ_{mem} is unable to do so. This novel piece of data supports the notion that NuMA $_{mem}$ is necessary for cortical dynein localization during anaphase. We note also that another glimpse into the biological relevance of the interaction of NuMA with the plasma membrane comes from our finding that PI3K inhibition causes PIP₂-dependent enrichment of cortical NuMA during metaphase, which results in spindle positioning defects (see Figure 7 and Supplementary Figure S6).

Minor comments:

- Page 13: PIP3 "... appears to be strictly in the cytoplasm ..."; it is equally or more likely that under the given condition cells have too little PIP3 in the membrane for it to be efficiently labelled by AKT-PH domains. I would recommend rephrasing the sentence.

Thank you for this suggestion. The text has been reworded accordingly.

Reviewer #2

This reviewer stated that ‘Overall, the experiments are convincing and the results are important for the field of spindle positioning and thus in general for cell division’ and felt that the “... article could be almost published as it is”, while conveying a few minor comments, which we have addressed as detailed below.

1) An important point made by the authors is that 4.1G/R in fact act on cortical actin, which is itself required for NuMA recruitment at the cell cortex. But the images showing the actin cortex in 4.1(R+G) RNAi cells do not show such a drastic perturbation of the actin cortex. Could the author show that using low doses of LatA which would have similar partial effect on actin, NuMA is already affected as much as with the 4.1(G+R) RNAi?

Response. We thank the reviewer for suggesting this experiment. We have now tested the consequence of a range of Latrunculin A concentrations (50 nM-1 μ M) and found that a 10 min treatment of mitotic cells with 200 nM Latrunculin A impairs the cortical actin cytoskeleton in a manner that resembles the consequence of 4.1(R+G) depletion. Importantly, we found in addition that such cells exhibit a concomitant diminution of NuMA/p150^{Glued} cortical localization that also resembles that provoked by depleting 4.1(R+G) proteins. These results are shown in the novel Supplementary Figure S4A-L and discussed on page 11 of the manuscript. Please note that although the actin cytoskeleton of cells treated with 200 nM Latrunculin A resembles that of cells depleted of 4.1(R+G) (compare Fig. S3K-N with Fig. S4C and S4F), it is not identical, with Latrunculin A treatment inducing a more patchy pattern of residual cortical F-actin. That this is the case is spelled out in the legend of Supplementary Figure S4.

2) The authors propose an alternative mechanism for the recruitment of NuMA, but they do not show that the perturbations they do on PiPs do not affect cortical actin. Similarly to the effect of 4.1 depletion, perturbing PiPs is very likely to affect cortical actin too (in particular the perturbation via Ionomycin and Ca²⁺ is almost certain to strongly affect cortical actin).

Response. We thank the reviewer for raising this possibility, which is in line with the notion that perturbation of PIPs influences the cortical actin cytoskeleton in

other systems (reviewed by Yin and Janmey, 2003; PMID: 12471164). To address this possibility, we set out to investigate the influence of Ionomycin and Ca^{2+} treatment on the cortical actin cytoskeleton, both in interphase and in mitotic cells. As shown in Supplementary Figure S5G and S6H, incubation with Ionomycin and Ca^{2+} drastically influences the actin cytoskeleton during interphase, with profound changes in internal stress fibers. By contrast, we found that such treatment does not influence the cortical actin cytoskeleton during mitosis. These new experiments are shown in Supplementary Figure S5G-J and discussed on p. 15 of the revised manuscript. Furthermore, we also investigated the consequence of Rapamycin-induced targeting of the hybrid lipid phosphatase pseudojanin (PJ) on the actin cytoskeleton. These experiments revealed that Rapamycin-induced targeting of PJ does not influence the cortical actin cytoskeleton during mitosis (see new Supplementary Figure S5M and S5N). Overall, these new experiments lead us to conclude that although affecting PIPs can influence the actin cytoskeleton in some settings, the treatments that were used in our work to alter PIP/PIP₂ levels do not perturb the cortical actin cytoskeleton during mitosis. See also point 3 of reviewer 3.

3) Alternatively, do the authors suggest that the effect of cortical actin on NuMA is through PiPs? How do they reconcile the need for an intact actin cortex for NuMA recruitment and the role of PiPs?

Response. Prompted by the reviewer's remark, we have tested if the localization of PIP₂ (as monitored with GFP-PLC δ -PH) is perturbed upon actin depolymerization. Although we noticed some deformation of the cells upon the addition of 1 μM Latrunculin A during 10 min, no other significant change in the cortical localization of PIP₂ was observed (compare the new Supplementary Figure S5K with S5L). Therefore, the impairment in cortical NuMA/p150^{Glued} localization observed upon Latrunculin A treatment (Supplementary Figure S4G-L) does not appear to be due to an impact on PIP₂. Overall, we conclude that while PIPs are essential for the presence of cortical anaphase NuMA, the actin cytoskeleton participates in promoting such localization in an independent manner. That this is the case is spelled out explicitly on p. 15-16 of the revised manuscript.

4) The authors did not describe what is the phenotype of having NuMA all over the cortex in anaphase. Is there an effect on spindle positioning or elongation?

Response. We explored the interesting question raised by the reviewer by

conducting live-imaging experiments of a few cells expressing mCherry-H2B and GFP- α -tubulin, monitoring chromosomes-chromosomes as well as pole-pole distances, but did not observe an apparent impact on spindle elongation upon CYK4 depletion. That this is the case is mentioned explicitly on p. 21 in the discussion of the revised manuscript. However, we think that this question would need to be analyzed in more depth to reach a definitive conclusion, including by devising ways to artificially target NuMA to the equatorial region in otherwise unperturbed cells.

5) The sentence on tumorigenesis in the discussion is a bit useless and could be removed.

Response. Agreed -the sentence has been removed from the revised manuscript.

6) The proposed mechanism of competition between CYK4 and NuMA for binding to PIPs is not very convincing. As they performed *in vitro* experiments for NuMA binding, maybe they could try their idea *in vitro*? (this is nevertheless absolutely not required for this article, as it is not a very central point).

Response. Time will tell whether the competition between CYK4 and NuMA occurs through the mechanism proposed on the basis of these initial experiments, and reconstituting such competition *in vitro* is certainly an interesting suggestion to consider. However, conducting such experiments appears to fall outside the scope of the present manuscript. Note also that we now discuss (p. 21-22 of the revised manuscript) that the postulated competition mechanism is likely to be more complex given notably that NuMA fragments containing the membrane binding region are not excluded from the equatorial region. See also responses to point 2 of reviewer 1 and to point 2 of reviewer 3.

Reviewer #3

This reviewer mentioned that “... *they (the authors) present a novel mechanism for a direct link between astral microtubules and the plasma membrane. In addition, the authors have made sufficient contribution to clarify inconsistency in previous studies*”. However, he/she requests that we address a few points before drawing the conclusions in the manner presented. How this has been achieved is explained below.

Section IA. Specific major concerns (Points for data clarification):

Does the localization of cortical Dynein mirror the localization of NuMA mutants in Figure 1? In the image of the mutant expressing cell (Figure 1L and 1Q), is the level of NuMA dramatically reduced at spindle poles and increased at cortex - if so, how about dynein localization? This is important to address to fully understand the underlying biological significance.

Response. We reported previously that the localization of wild-type NuMA or NuMA_{T2055A} in both metaphase and anaphase goes hand in hand with that of cortical dynein (Kotak et al., 2013; PMID: 16085494). We had also reported that metaphase cells treated with the CDK1 inhibitor RO-3306 or expressing GFP-NuMA_{T2055A} exhibit an increase of cortical NuMA and dynein, and a concomitant decrease in the pools of these proteins residing at spindle poles (Kotak et al., 2013; PMID: 16085494). These findings make us confident that the localizations of GFP-NuMA fusion proteins shown in Figure 1H reflect that of dynein. Nevertheless, as requested by the reviewer, we have determined the distribution of the dynein complex component p150^{Glued} in cells expressing GFP-NuMA_{Δ4.1,T>A}. As anticipated, we found a dramatic enrichment of p150^{Glued} at the cell cortex and a concomitant decrease of the protein pool at the spindle in metaphase. This new experiment is shown in the Supplementary Figure S4P-S4S and discussed on p. 12 of the text.

2. In Figure 2C, is the NuMA (mem+C-ter) mutant excluded from the equatorial cell-cortex or not? The image shown looks ambiguous. This is important to conclude the domain responsible for NuMA exclusion from equatorial cortex.

Response. We thank the reviewer for making this important observation. Prompted by this comment, we analyzed carefully the distribution of the various

fusion constructs, and found that cells expressing GFP-NuMA_{mem+C-ter} indeed exhibit GFP signal in the equatorial cortical region, in contrast to cells expressing full length GFP-NuMA (compare Figure 3B with 3D). Presence in the equatorial cortical region was also observed in cells expressing GFP-NuMA_{mem}. That this is the case is spelled out on p. 44 in the legend of this figure, and elaborated on some more on p. 21 of the discussion section. See also responses to point 1 of reviewer 1 and to point 6 of reviewer 2.

3. Is it possible that altering phosphoinositide levels within the cell or cell-cortex disrupts cortical actin function and in turn, indirectly abrogates NUMA recruitment? If this is technically difficult to disentangle, and not the scope of this paper, the authors should at least discuss this possibility because they are using an argument of altered actin function to negate some of the interpretations made by the Kiyomitsu and Cheeseman 2013 Cell paper and this could be true for their own model as well.

Response. We thank the reviewer for raising this possibility, which is in line with the notion that perturbation of PIPs influences the cortical actin cytoskeleton in other systems (reviewed by Yin and Janmey, 2003; PMID: 12471164). To address this possibility, we set out to investigate the influence of Ionomycin and Ca²⁺ treatment on the cortical actin cytoskeleton, both in interphase and in mitotic cells. As shown in Supplementary Figure S5G and S6H, incubation with Ionomycin and Ca²⁺ drastically influences the actin cytoskeleton during interphase, with profound changes in internal stress fibers. By contrast, we found that such treatment does not influence the cortical actin cytoskeleton during mitosis. These new experiments are shown in Supplementary Figure S5G-J and discussed on p. 15 of the revised manuscript. Furthermore, we also investigated the consequence of Rapamycin-induced targeting of the hybrid lipid phosphatase pseudojanin (PJ) on the actin cytoskeleton. These experiments revealed that Rapamycin-induced targeting of PJ does not influence the cortical actin cytoskeleton during mitosis (see new Supplementary Figure S5M and S5N). Overall, these new experiments lead us to conclude that although affecting PIPs can influence the actin cytoskeleton in some settings, the treatments that were used in our work to alter PIP/PIP₂ levels do not perturb the cortical actin cytoskeleton during mitosis. See also point 2 of reviewer 2.

4. How are spindle oscillations calculated and how are they distinguished from tumbling? In the time-lapse sequence presented in Figures 5 and S4 and

associated movies, spindle tumbling, but not oscillation, is observed. It will be useful to measure pole-to-pole oscillation as a kymograph to compare oscillations across conditions. Also, statistical significance of differences between the s.e.m error bars in Figures 5E-G should be indicated using p-values (preferably shown as SD bars as in other figures).

Response. We apologize for the misunderstanding, which probably stems from us mistakenly not having included a section on how spindle oscillations were determined in the original manuscript. This information has been included in the revised manuscript (p. 28 and 48-49). In a nutshell, the bar graphs are readouts of the extent of spindle oscillations, representing the frequency at which chromosome position changes $>10^\circ$ between two frames, a metric that we have used previously to quantify such movements and which we would favor sticking to in the present manuscript for consistency (Kotak et al., 2013; PMID: 16085494; Kotak et al., 2012; PMID: 23027904). The requested change from showing SEM to showing SD has been implemented, and the p value is now reported (p. 49 of the revised manuscript).

Section IB. Specific major concerns (Points for text clarification):

1a. Results presented in the main Figure 1A-F are already known. It is already established that the LGN/Galpha platform is dispensable for cortical dynein recruitment in anaphase (eg., Kiyomitsu and Cheeseman, 2013). The authors could briefly clarify as to why they are presented as novel findings/ how these results extend current findings.

Our initial writing reflected the fact that we made this observation before the Kiyomitsu and Cheeseman paper was published (as can be gleaned from the review process file of Kotak et al., 2013; PMID: 16085494). However, the reviewer is obviously correct in pointing out that this aspect of the work is no longer novel. We rectified the text to spell out clearly what aspect of our work is merely confirmatory of previous findings and what is truly novel at the present time (p. 6).

1b. Along the same lines, why do the authors consider their findings 'unexpected' in the discussion statement below: "Unexpectedly, we found here that the ternary complex components LGN/Gai1-3 are dispensable for NuMA-dependent enrichment of cortical dynein in the polar regions during anaphase"

What we meant to convey here is that this finding is unexpected in the light of the fact that LGN/Gai₁₋₃ were assumed to be the sole cortical anchor for NuMA/dynein (and not that it was unexpected in the light of the Kiyomitsu and Cheeseman paper). This point has been clarified in the revised manuscript (p. 6).

2. Some of the panels in Figure-1 are not described in results text (see page 7) and it's confusing to see these panels referred to in the later part of the manuscript.

We see the point raised by the reviewer. However, on balance, we find that the current layout is preferable because it allows us to maximize space utilization in the figures.

3. I have two concerns regarding this statement: "In addition to uncovering a switch in the mechanism directing NuMA/dynein to the polar regions of the cell between metaphase and anaphase, we discovered an accompanying switch in the mechanism preventing their accumulation in the cortical equatorial region."

3a. Are the authors referring to their previous paper when they mention "In addition to uncovering a switch in the mechanism directing NuMA/dynein to the polar regions of the cell between metaphase and anaphase"? If yes, this should be referenced and made clear.

We apologize for being insufficiently clear here: we meant to be referring to the present manuscript and to the switch in the anchoring mechanism between metaphase (LGN/Gai₁₋₃ dependency) and anaphase (PIP/PIP₂ dependency). The wording has been altered to clarify this point (p. 19-20).

3b. The statement "we discovered an accompanying switch in the mechanism preventing their accumulation in the cortical equatorial region" is not fully supported here for the following reasons: The authors simply have a correlation between loss of CYK4 and accumulation of NUMA at equatorial cortex; and as they report they have a 'tempting possibility' that the two proteins may compete for same lipid moieties. If competition is indeed the full explanation for the switch, one would expect that NUMA overexpression displaces CYK4 from the equatorial cortex, but this has not been shown. Therefore, the sentence should be reworded as a proposal, rather than as discovery of a switch mechanism.

The reviewer is correct in pointing out that the postulated competition mechanism is likely to be more complex than what we had envisioned initially. In line with this remark, we found that overexpression of full length GFP-NuMA does not cause

apparent cytokinesis abnormalities. This suggests that excess NuMA is not sufficient to displace CYK4 from the equatorial region, perhaps because CYK4 has a higher local concentration and/or higher affinity towards phospholipids. Regardless of the actual reason, we agree with the reviewer that the present understanding of this question calls for more balanced wording, which we have implemented on p. 21-22 in the revised manuscript.

4. Toyoshima et al., 2007 reported metaphase-positioning defects in response to impaired PI3K activity. In this paper, phosphoinositides are reported to be responsible for anaphase recruitment of NuMA to cortex. Is there an LGN-Galpha independent pool of NuMA at the metaphase cell cortex? If so, could this be observed in Figures 1A-F?

We did not observe an LGN/Gai-independent pool of NuMA in normal metaphase cells, most likely because of the limited pool of unphosphorylated NuMA that is available at that stage of the cell cycle. However, acute CDK1 inactivation results in the presence of excess nonphosphorylated NuMA, which can localize at the plasma membrane independently of LGN/Gai, as shown in the Supplementary Figure S1P and S1Q and in the related text on p. 7.

Section II: Minor concerns that should be addressed

(i) Scale bars are missing in several images (examples: FigureS1, S3)

Thanks for pointing this out, this has been corrected.

(ii) N values missing (examples: Figures 3, S4)

Apologies –this has been taken care of.

(iii) Typos: Page 5: 'indentify' instead of 'identify',

Corrected.

(iv) inconsistent nomenclature for s.e.m, Galphai(1-3)

Sorry for this mistake, which has been fixed.

(v) Incomplete sentence in methods:

"CDK1 inhibition was performed by treating metaphase synchronized cells for 5 minutes with either RO-3306 (Vassilev et al, 2006) (9 μ M; Santa Cruz, sc-358700)"

Corrected as well.

2nd Editorial decision

28 May 2014

Thank you for submitting your revised manuscript on NuMA phosphoinositide interaction for our consideration. The original reviewers have now looked at it once more, and I am pleased to inform you that all of them are satisfied with the revisions and consider the study now suitable for publication in The EMBO Journal.

Before proceeding with formal acceptance of the manuscript, I would like to just ask you for a few minor modifications that you may send via email in a revised manuscript text file:

Please take care of the minor corrections still asked for by referee 1. In addition, should you have any quantitative analyses supporting figures S3 and S4, you may want to include them too as asked by this referee, but I would not consider this essential at this point

After these final modifications, we should be able to swiftly proceed with formal acceptance and production of the manuscript. Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication!

Referee #1

In the revised version the authors have included a series of additional experiments and analyses that strengthen the main conclusions of the work and that have addressed most of the key points raised by this and by other reviewers. The work provides a conceptual advance by showing that NuMA can contact the plasma membrane to link the mitotic spindle to the membrane in anaphase cells. This reviewer is supportive of publication of the revised manuscript.

One point the authors could improve on before publication would be to provide a quantitative analysis of the actin cortex phenotype shown in Figure S3 and S4. Based on the pictures provided it is hard to judge.

Minor points to be corrected:

- Page 10: "...of such abolition..." should be "... abolishing such"
- Page 13: "... polyanionic phosphoinositide ..." should be plural
- Page 14: "By contrast to" should be "In contrast to ..."
- Figure 4B: "SE" should be "PS"

Referee #2

This revised version of the manuscript contains a large number of new experiments which address all the points I raised. I also think that they addressed well the points raised by the other referees; in particular my main concern was that the actin cortex could also be affected when PiPs were affected. It is a bit surprising that it is not the case, but the authors should it quite clearly. I thus think that the article should be published as it is.

Referee #3

The authors have addressed my main concerns, and I am happy to recommend publication in EMBO J.